







# The aorta can act as a site of naïve CD4<sup>+</sup> T-cell priming

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<b>Aims</b>	Aortic adaptive immunity plays a role in atherosclerosis; however, the precise mechanisms leading to T-cell activation in the arterial wall remain poorly understood.
<b>Methods and results</b>	Here, we have identified naïve T cells in the aorta of wild-type and T-cell receptor transgenic mice and we demonstrate that naïve T cells can be primed directly in the vessel wall with both kinetics and frequency of T-cell activation found to be similar to splenic and lymphoid T cells. Aortic homing of naïve T cells is regulated at least in part by the P-selectin glycosylated ligand-1 receptor. In experimental atherosclerosis the aorta supports CD4 <sup>+</sup> T-cell activation selectively driving Th1 polarization. By contrast, secondary lymphoid organs display Treg expansion.
<b>Conclusion</b>	Our results demonstrate that the aorta can support T-cell priming and that naïve T cells traffic between the circulation and vessel wall. These data underpin the paradigm that local priming of T cells specific for plaque antigens contributes to atherosclerosis progression.
<b>Keywords</b>	Aorta • Atherosclerosis • Priming • T cells

## 1. Introduction

Aortic adaptive immune responses play a role in atherosclerosis,<sup>1</sup> with several immune cell subsets identified in human<sup>2,3</sup> and murine<sup>4,5</sup> vessels; however, to date the precise mechanisms leading to T-cell activation in the arterial wall remain poorly understood.

Several studies have investigated whether vascular resident antigen presenting cells (APCs) retain the ability to present antigen locally to T cells using reductionist approaches such as adoptive transfer of model antigen loaded dendritic cells (DCs),<sup>6</sup> *in vitro* co-culture systems,<sup>7</sup> and explanted aortas.<sup>8</sup> Building on these approaches, antigen presentation has been demonstrated *in vivo* by constitutive aortic plasmacytoid DCs (pDCs).<sup>9,10</sup> More recently, it was discovered that in the advanced stages

of atherosclerosis in apoE<sup>-/-</sup> mice, the vessel wall orchestrates the formation of artery tertiary lymphoid organs that control vascular T-cell responses with concomitant reduction in adjacent plaque size without involvement of secondary lymphoid organs (SLOs).<sup>4</sup> Therefore, current evidence suggests that aortic APCs possess the capacity to present antigen and that the aorta may act as a site of T-cell priming in atherosclerosis.

In earlier stage pathology, it is assumed that adaptive immune responses are co-ordinated in SLOs and/or the vessel wall, but naïve T cells have yet to be identified to reside constitutively in vascular tissue and it remains controversial as to whether T-cell priming can occur within the aorta. Therefore, in this investigation, we set out to address the following questions: (i) Do naïve CD4<sup>+</sup> T cells reside in the aorta

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and by what mechanism are they recruited?; (ii) Can T-cell priming occur directly in the aorta and where do T-cell/DC contacts take place?; and (iii) How does the aortic CD4<sup>+</sup> T-cell phenotype compare with T cells in SLOs and are there divergent effects on these distinct populations after induction of pathology?

Our results demonstrate that the aorta can act as a site of naïve CD4<sup>+</sup> T-cell priming and selectively induce a localized Th1 immune response in early experimental atherosclerosis.

## 2. Methods

### 2.1 Animals

B6.129P2-Apoe(tm1Unc)/J (apoE<sup>-/-</sup>) mice, C57BL/6 (wild type; WT), OT-II, and TEa mice were used in this study. All animals were euthanized by carbon dioxide. No anaesthetic agent was used. For full details see [Supplementary material online](#). All the procedures were performed in accordance with local ethical and UK Home Office regulations and conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

### 2.2 Flow cytometry

Cell suspensions from aortas were prepared by enzyme digestion as previously described.<sup>4,9</sup> For experiments that involved removal of aortic adventitia, a modified digestion protocol was performed to allow removal of adventitia.<sup>11</sup> Spleens and renal lymph nodes (rLNs; abdominal aortic draining) were digested in collagenase D (Sigma-Aldrich, Irvine, UK). Intracellular staining was performed using a cytofix/cytoperm kit (BD Biosciences, Oxford, UK) or a True-Nuclear™ Transcription factor buffer set (Biolegend, London, UK) according to the manufacturer's instructions. For BrdU staining, a BD Pharmingen FITC BrdU flow kit (BD Biosciences) was used according to the manufacturer's instructions. Because T cells represent a major population (~20%) of blood leucocytes coupled with the fact that aortic naïve T cells were likely to be rare, we included an additional control for all T-cell experiments to ensure that T cells analysed were 100% aortic resident: 3 min prior to the experiment endpoint, animals were injected i.v. with a CD45.1 antibody (OT-II mice) or a CD45.2 antibody (WT, TEa, and apoE<sup>-/-</sup> mice) to label circulating blood leucocytes for subsequent exclusion from analysis ([Supplementary material online, Figure S1B](#)). Experiments were analysed on a LSR II, LSRFortessa (BD Biosciences) or a MACSQuant Analyzer (Miltenyi Biotec, Bisley, UK) using FlowJo (FlowJo LLC, Ashland, OR, USA). For full details and antibodies used see [Supplementary material online](#).

### 2.3 Assessment of antigen-induced T-cell proliferation *in vivo*

In one set of experiments, OT-II mice were injected i.v. with 200 µg chicken ovalbumin (OVA) and culled at 24, 48, or 72 h later. Phosphate-buffered saline (PBS) served as naïve control. Two hours prior to culling, mice were injected with 1 mg BrdU i.p. Single cell suspensions were prepared for aorta, rLNs, and spleen, and CD4<sup>+</sup> OT-II+ T cells stained for proliferation markers: BrdU and Ki-67 as described in [Supplementary material online](#).

In a separate series of experiments, OT-II mice were injected with 1 mg/kg FTY720 (Sigma-Aldrich) and 2 mg/kg anti-P-selectin (RMP-1) and anti-E-selectin (RME-1) antibodies (Biolegend) to block T-cell vascular recruitment, prior to receiving 200 µg OVA. Treatment with FTY720

and anti-P-selectin/anti-E-selectin was repeated on Day 2 (24 h following first dose) to maintain effective plasma concentrations. The control group received 200 µg OVA in addition to respective vehicle (dH<sub>2</sub>O) and isotype control antibodies (mouse IgG1, k and mouse IgG2a, k). Mice were culled 48 h following OVA administration ([Supplementary material online, Figure S2](#)). Single cell suspensions of aorta, rLNs, and spleen were obtained for flow cytometry analysis of CD4<sup>+</sup> OT-II proliferation as assessed by Ki-67.

### 2.4 Blockade of aortic T-cell recruitment

WT mice were treated with 100 µg anti-P-selectin glycosylated ligand (PSGL-1; 4RA10) or equivalent dose of corresponding isotype (Bio X Cell, West Lebanon, NH, USA) at 0 and 24 h to block aortic T-cell recruitment. Mice were culled at 48 h after first dose. Aortic single cell suspensions were obtained for flow cytometry quantification of total CD45<sup>+</sup> cells, CD4<sup>+</sup> T cells, and naïve CD4<sup>+</sup> T cells.

### 2.5 Assessment of Th1 cytokine expression

WT and apoE<sup>-/-</sup> mice were injected i.p. with 300 µg Brefeldin A (Sigma-Aldrich) to block cytokine release and culled 5 h later. Aortas, spleens, and rLNs were harvested and stained for intracellular cytokines via flow cytometry as detailed in [Supplementary material online](#).

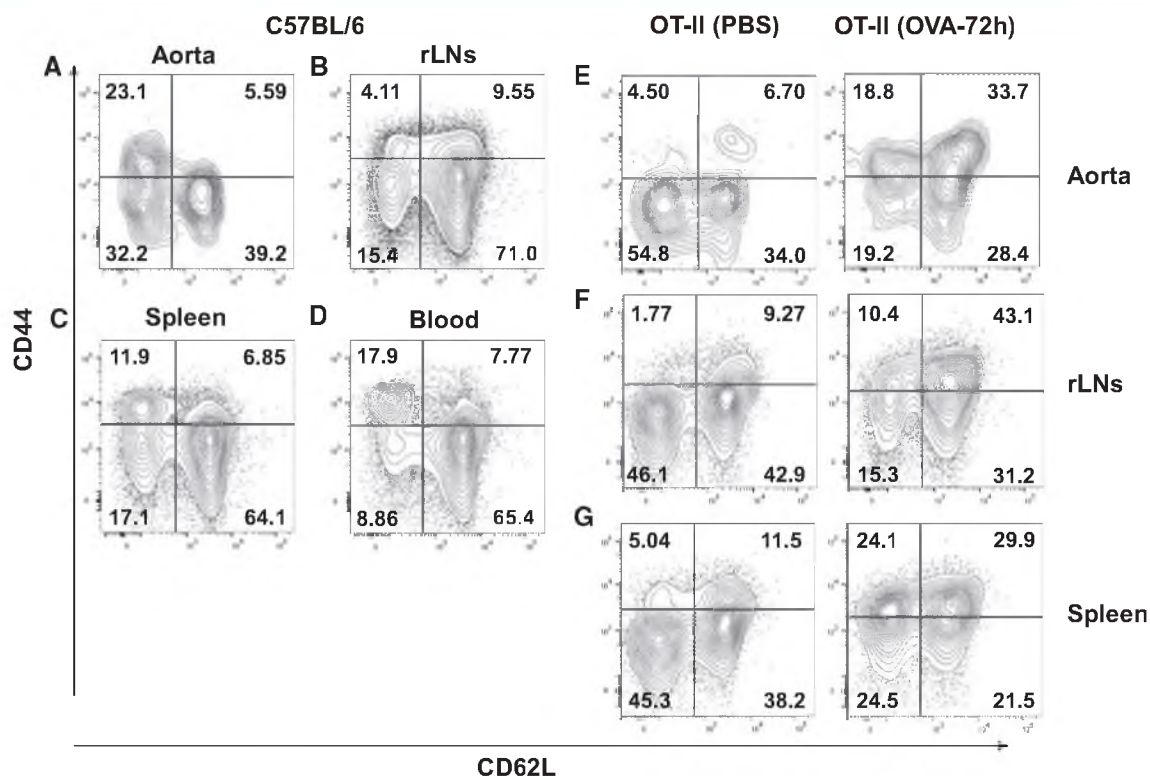
### 2.6 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) of *n* animals/groups of pooled tissues for each experiment. The Student's *t*-test was used to compare two groups. Analysis of variance (ANOVA) was used for comparing three or more groups with Tukey's multiple comparison post-test being applied as described in Figure legends. GraphPad Prism 6 software (San Diego, CA, USA) was used. A *P*-value <0.05 was taken to indicate statistical significance.

## 3. Results

### 3.1 Naïve CD4<sup>+</sup> T cells exist constitutively in the adventitia and intima/medial layers of WT aorta

The combination of perfusion and removal of extra-aortic tissue resulted in a pure vascular preparation ([Supplementary material online, Figure S1](#)). To determine the constitutive repertoire of T cells that reside in the whole naïve aorta, we employed high resolution cytometry by time of flight (CyTOF) which revealed multiple distinct CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations, two γδ populations and unidentified T-cell subtypes ([Supplementary material online, Figure S3](#)). Inspection of this data reveals that the aorta harbours a mix of CD44<sup>low</sup> and CD44<sup>hi</sup> T cells ([Supplementary material online, Figure S3](#)). Using flow cytometry, we found ~40% of aortic CD4<sup>+</sup> T cells expressed the classical naïve phenotype: CD62L<sup>+</sup>CD44<sup>lo</sup> ([Figure 1A](#)). This compares with approximately two-thirds of CD4<sup>+</sup> T cells being naïve in renal lymph nodes (rLNs), spleen, and blood ([Figure 1B–D](#)). Further characterization demonstrated that a clear CD4<sup>+</sup> T-cell population could be observed both within the adventitia and intima/media ([Supplementary material online, Figure S4B](#)). Analysis of the T-cell population revealed naïve CD4<sup>+</sup> T cells in both compartments but with a higher proportion of activated T cells (CD44<sup>hi</sup>) being present within the adventitia ([Supplementary material online, Figure S4C](#)). T cells were also visualized by confocal microscopy and found to exist in both the intima and adventitia of the naïve aorta



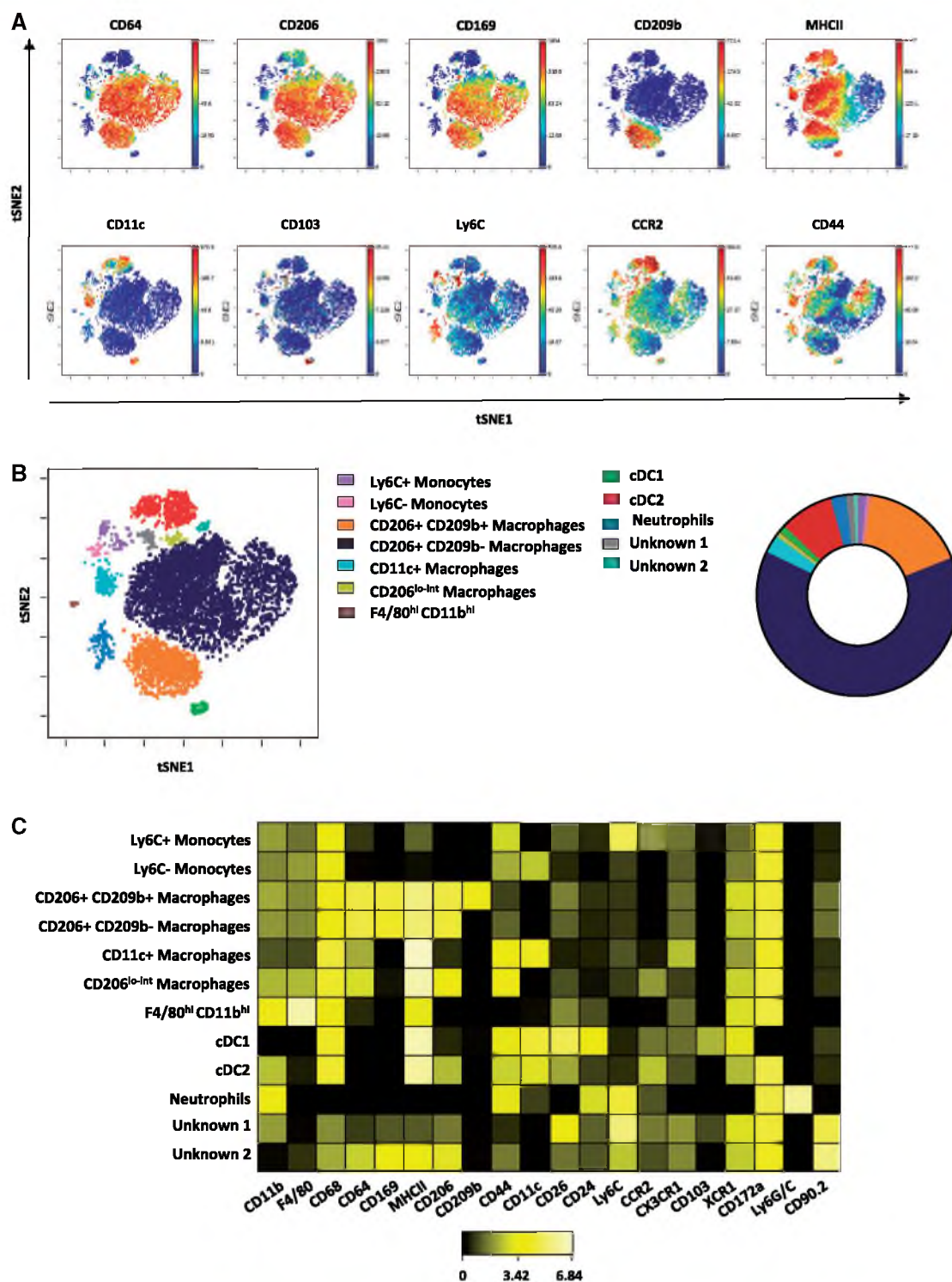
**Figure 1** Naïve T cells constitutively reside in C57BL/6 wild-type aorta and are reduced in OT-II murine aorta following antigen administration. (A–D) C57BL/6 wild-type mice were culled and single cell suspensions prepared for flow cytometry analysis of (A) aorta, (B) renal lymph nodes, (C) spleen, and (D) blood. CD4<sup>+</sup> T cells were stained with CD62L and CD44 to distinguish naïve from activated cells. All plots are gated on CD4<sup>+</sup>TCR-β<sup>+</sup> T cells. Results are from two independent experiments, with each group containing five-pooled aortas. (E–G) In a separate experiment, OT-II mice were injected with ovalbumin or PBS and culled 72 h later. Single cell suspensions prepared for flow cytometry analysis of (E) aorta, (F) renal LNs, and (G) spleen were stained with CD62L and CD44 to once again distinguish naïve from activated cells. All plots are gated on CD4<sup>+</sup>Vα2<sup>+</sup>Vβ5<sup>+</sup>. Results are from two independent experiments, with each group containing five-pooled aortas. Spleen, LNs, and blood were analysed individually.

including T cells bound to the aortic endothelium (Supplementary material online, Figure S7A). The presence of naïve T cells within peripheral tissues remains a matter of controversy. To further validate our finding that the aortic CD62L<sup>+</sup>CD44<sup>-</sup> cells are truly naïve, we employed TEa mice that consist of a CD4<sup>+</sup> T-cell population expressing a single T-cell receptor (TCR) specificity and are also RAG deficient, thus T cells remain naïve in the absence of Eα52–68: I-Ab complex (their cognate antigen). By employing this model, we are able to determine if naïve T cells are capable of trafficking to and maintaining a clear resident population within the aorta, even under non-inflammatory conditions. This was indeed what we observed: In TEa mice, the vast majority of CD4<sup>+</sup> T cells in the aorta are CD62L<sup>+</sup>CD44<sup>-</sup>, conclusively demonstrating that naïve CD4<sup>+</sup> T cells do indeed reside within the aorta (Supplementary material online, Figure S5A). Data from renal lymph nodes are presented for comparison where similarly, most CD4<sup>+</sup> T cells were CD62L<sup>+</sup>CD44<sup>-</sup> naïve cells (Supplementary material online, Figure S5B). The aorta, therefore, acts as a reservoir of naïve CD4<sup>+</sup> T cells that may form interactions with aortic resident antigen presenting myeloid cells. To fully delineate the repertoire of myeloid cells within the naïve aorta, we performed CyTOF analysis of C57BL/6 aortas which revealed 12 types of myeloid cells including multiple major histocompatibility complex Class II (MHC-II<sup>hi</sup>) populations consisting of distinct cDC and macrophage subsets (Figure 2).

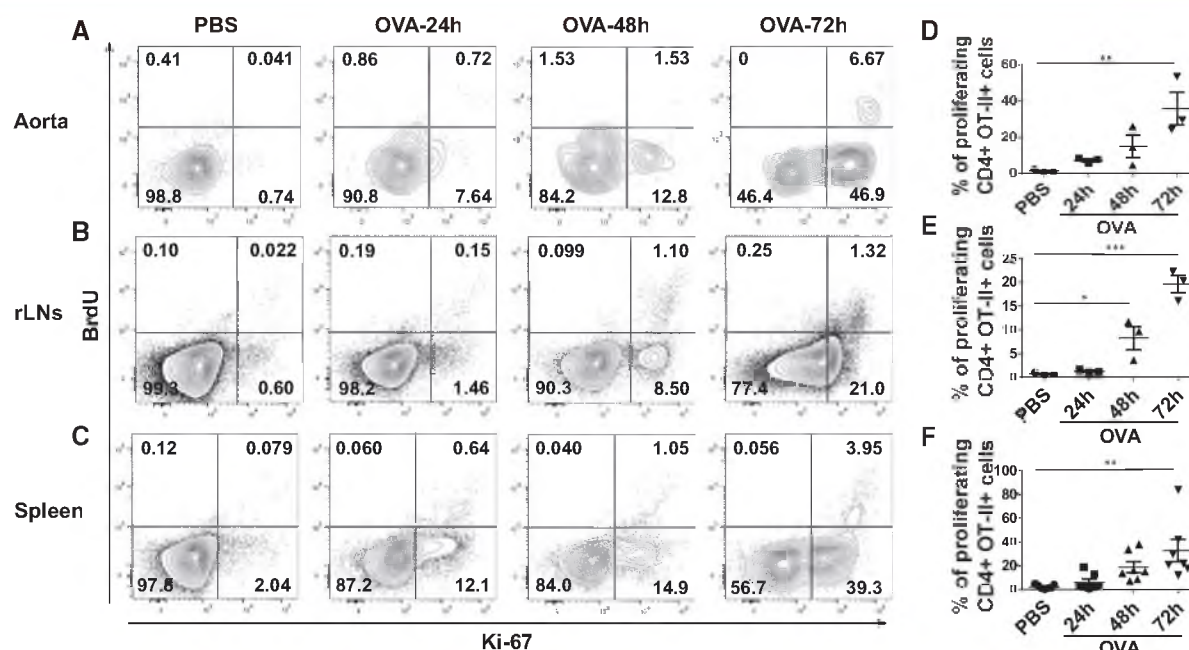
### 3.2 Priming of CD4<sup>+</sup> T cells occurs within the aorta

Evidence exists that T-cell activation occurs within the aorta,<sup>6,8,12</sup> but *in vivo* proof of naïve T-cell priming is lacking. Conventional approaches of assessing naïve T-cell homing and antigen-dependent activation via adoptive transfer and subsequent priming in recipient mice is not feasible given that recruitment of donor lymphocytes to the naïve aorta make up only a minor fraction of the total aortic lymphocyte population.<sup>6</sup> Therefore, we adopted the method of only investigating endogenous T-cell homing and priming using OT-II mice. The use of OT-II mice, where the majority of CD4<sup>+</sup> T cells have a TCR specific for chicken ovalbumin (OVA) 323–339 in the context of I-Ab (alloantigen of H-2b bearing mouse strains, including mice on C57BL/6 background), allows us to assess whether the aorta can act as a site of priming by injecting OT-II mice with OVA so that the great majority of aortic CD4<sup>+</sup> T-cell-DC contacts will involve T cells with a TCR specific for the OVA peptide presented by aortic cDCs. PBS-treated OT-II aortas contained a similar proportion of naïve CD4<sup>+</sup> T cells to WT mice (Figure 1E). Following administration of antigen, there was a switch towards a more activated CD44<sup>hi</sup> phenotype with a concomitant reduction in naïve T cells (Figure 1E). This was also true for rLNs and spleen (Figure 1F and G) indicating each site harbours an antigen-activated CD4<sup>+</sup> population in parallel with a reduced frequency





**Figure 2** Mass cytometry reveals myeloid populations in C57BL/6 aorta. (A) Myeloid cells were gated as Lin-CD11b<sup>lo-hi</sup> and clustered using viSNE on the expression of cell surface and intracellular markers. Expression levels of selected myeloid markers in the resulting viSNE clustered cell populations is shown for a representative C57BL/6 mouse. (B) Twelve cell populations consisting of monocytes (Ly6C<sup>+</sup> and Ly6C<sup>-</sup>), conventional Types 1 and 2 dendritic cells (cDC1 and cDC2), neutrophils, five macrophage subsets, and two unidentified populations. Doughnut plot shows mean proportion of subsets in aortas of  $n = 6$  mice. (C) Heatmap showing the relative expression level of 20 cell markers within the 12 myeloid cell subsets identified by the viSNE clustering shown in B.

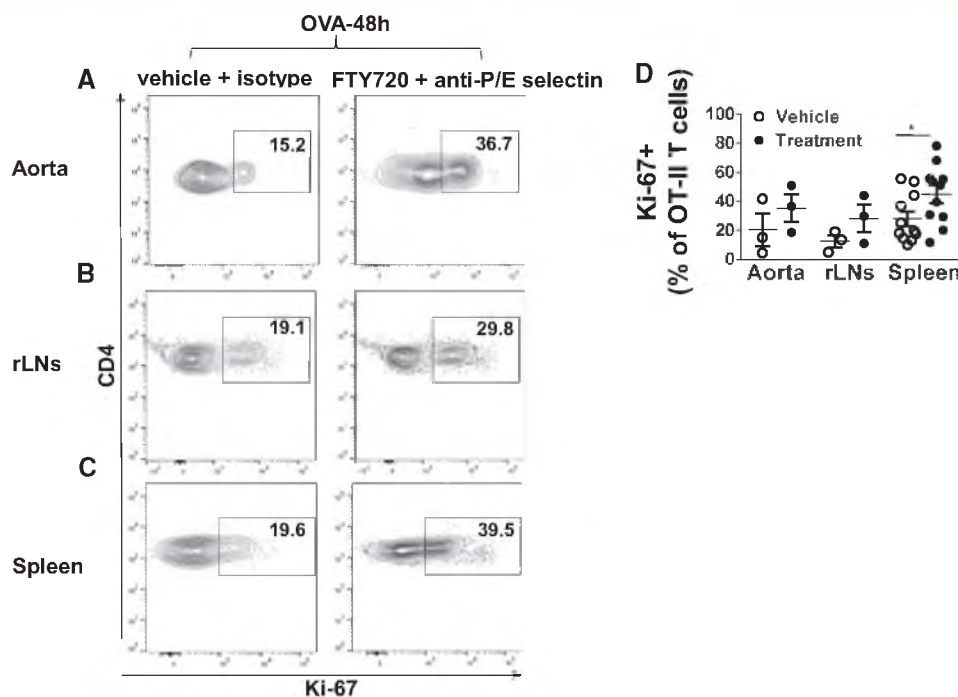


**Figure 3** Kinetics of antigen-dependent T-cell proliferation is similar in aorta compared to secondary lymphoid organs. OT-II mice were injected with 200  $\mu$ g OVA and culled at 24, 48, or 72 h later. PBS served as naïve animal controls. Two hours prior to culling, mice were injected with 1 mg BrdU. Single cell suspensions were prepared for aorta, renal LNs, and spleen, and CD4<sup>+</sup> OT-II T cells stained for proliferation markers: BrdU and Ki-67. Representative plots for T-cell proliferation markers are shown for (A) aorta, (B) renal LNs, and (C) spleen. Frequency of proliferating CD4<sup>+</sup> OT-II T cells are graphically displayed for (D) aorta, (E) renal LNs, and (F) spleen. Results represent three independent experiments with aortas and renal LNs from three mice pooled per group with spleens analysed individually. Individual data points represent average value per group/spleen; horizontal bars denote mean. Results are presented as mean  $\pm$  SEM. One-way ANOVA followed by Tukey's *post hoc* correction; \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 vs. PBS.

of naïve T cells. Next, we sought to compare the kinetics of naïve T-cell activation in the aorta, rLNs, and spleen. To do this, we performed a time-course experiment in OT-II mice injected with OVA using the proliferation marker Ki-67. Additionally, we injected the animals with 1 mg BrdU 2 h prior to culling to measure cells actively undergoing DNA synthesis (S phase). A short BrdU pulse was employed to confine positive detection to cells actively replicating locally in the tissue of interest and to eliminate the contribution of cells that may have acquired BrdU in another location and then migrated to the aorta. Following treatment with OVA, the first Ki-67<sup>+</sup> cells emerged at 24 h. All three compartments displayed broadly similar kinetics in terms of OT-II T-cell proliferation with the aorta (Figure 3A) and spleen (Figure 3C) paralleling each other most closely. The first BrdU<sup>+</sup> cells become apparent in the aorta at 72 h, as observed for the spleen and OT-II<sup>+</sup> T-cell proliferation (Ki-67<sup>+</sup> cells) was significantly greater at 72 h compared with untreated animals in all three tissues analysed (Figure 3D–F). To summarize, we have shown the existence of naïve CD4<sup>+</sup> T cells within WT, TEa, and OT-II murine aorta. Moreover, the local vascular T-cell population becomes activated and undergoes proliferation following systemic administration of a model antigen.

To demonstrate aortic T-cell priming arises from a constitutive aortic resident T-cell population, we set out to block T-cell recirculation and blood derived T-cell binding/extravasation to the aorta utilizing FTY720 [functional antagonist on sphingosine 1-phosphate 1 (S1P<sub>1</sub>) receptors causing internalization of S1P<sub>1</sub> receptors on T cells which sequesters lymphocytes in SLOs] to deplete the blood of T cells and blocking

antibodies to P-selectin and E-selectin (RMP-1 and RME-1, respectively). FTY720 is highly efficient at sequestering T cells into SLOs and subsequently inducing lymphopenia in the blood.<sup>4,13,14</sup> Leucocyte binding and rolling on endothelium is critically dependent on P-selectin with a lesser contribution from E-selectin,<sup>15–17</sup> thus our rationale is that following this treatment protocol followed by antigen challenge in OT-II mice (Supplementary material online, Figure S2), we can confine T-cell priming to aortic resident cells only. Firstly, to determine the effect of FTY720 (in the presence of our blocking antibodies: RMP-1 and RME-1) on circulating blood CD4<sup>+</sup> T cells, we treated mice over the course of 2 days (FTY720 + RMP-1/RME-1) at 0 and 24 h and quantified CD4<sup>+</sup> T cells at 48 h. This resulted in a 93% reduction in circulating CD4<sup>+</sup> T cells (Supplementary material online, Figure S6A). One factor that could confound interpretation of our results is that FTY720 can also deplete T cells from peripheral tissues.<sup>18</sup> Therefore, we assessed whether naïve T cells still reside in the aorta following FTY720 + RMP-1/RME-1. Following treatment, there was a reduction in aortic CD4<sup>+</sup> T cells, likely due to impaired trafficking. While the frequency of naïve CD4<sup>+</sup> T cells was less than we had observed in untreated WT and OT-II mice previously, naïve T cells still resided in the aorta at our experiment endpoint (48 h) (Supplementary material online, Figure S6B) clearly indicating that naïve T cells would be available in the aorta for contact with OVA peptide carrying APCs (contacts that occur within the first 24 h following antigen). We studied 48 h as the earliest time point where a clear, distinct population of proliferating OT-II T cells was observed within the aorta of OT-II mice as shown in Figure 3A. As hypothesized, OT-II proliferation in



**Figure 4** T-cell proliferation following antigen is maintained in the aorta following blockade of lymphocyte trafficking and extravasation. OT-II mice were injected with 1 mg/kg FTY720 (or vehicle) and 2 mg/kg anti-P-selectin and anti-E-selectin antibodies (or isotype) 2 and 1 h, respectively prior to received 200 µg ovalbumin. Treatment with FTY720 and antibodies was repeated on Day 2 (24 h following first dose) to maintain effective plasma concentrations and mice were culled 48 h following ovalbumin administration. Single cell suspensions of aorta, renal LNs, and spleen were obtained for flow cytometry analysis of CD4<sup>+</sup> OT-II<sup>+</sup> T-cell proliferation as assessed by Ki-67. Representative plots illustrating T-cell proliferation for (A) aorta, (B) renal LNs, and (C) spleen. (D) Frequency of CD4<sup>+</sup> OT-II<sup>+</sup> T cells undergoing proliferation for each tissue. Results represent 3 independent experiments with 3–4 aortas and 3–4 sets of renal lymph nodes pooled per group with spleens analysed individually ( $n = 11$ ). Individual data points represent average value per group/spleen; horizontal bars denote mean. Results are presented as mean  $\pm$  SEM. The Student's unpaired  $t$ -test;  $*P < 0.05$ .

the treatment group was maintained at a level similar to the vehicle group indicating that OT-II cells undergoing proliferation following OVA were truly aortic resident (Figure 4).

To determine the location of DC-T-cell interactions within the aortic wall, OT-II mice were treated with OVA or PBS and culled 20 h later in order to visualize stable antigen-dependent contacts between DCs and T cells (Supplementary material online, Figure S7). As noted above, T cells resided in both the intima and adventitia and were sparsely scattered as single cells throughout the aortic tree. As expected, DCs were most notably located within the intima, lining the endothelium, and within the subendothelial space. DCs were also located deep within the media layer residing between smooth muscle fibres (Supplementary material online, Figure S7B). Following administration of OVA, T cells, and DCs showed co-localization with DC-T cells contacts observed in the intima, adventitia and within the media (Supplementary material online, Figure S7C). DC-T-cell contacts were not observed in PBS-treated mice.

In summary, these results clearly demonstrate that OT-II T cells are being primed locally within the vessel wall.

### 3.3 Naïve T-cell recruitment to the aorta is suppressed by blockade of PSGL-1

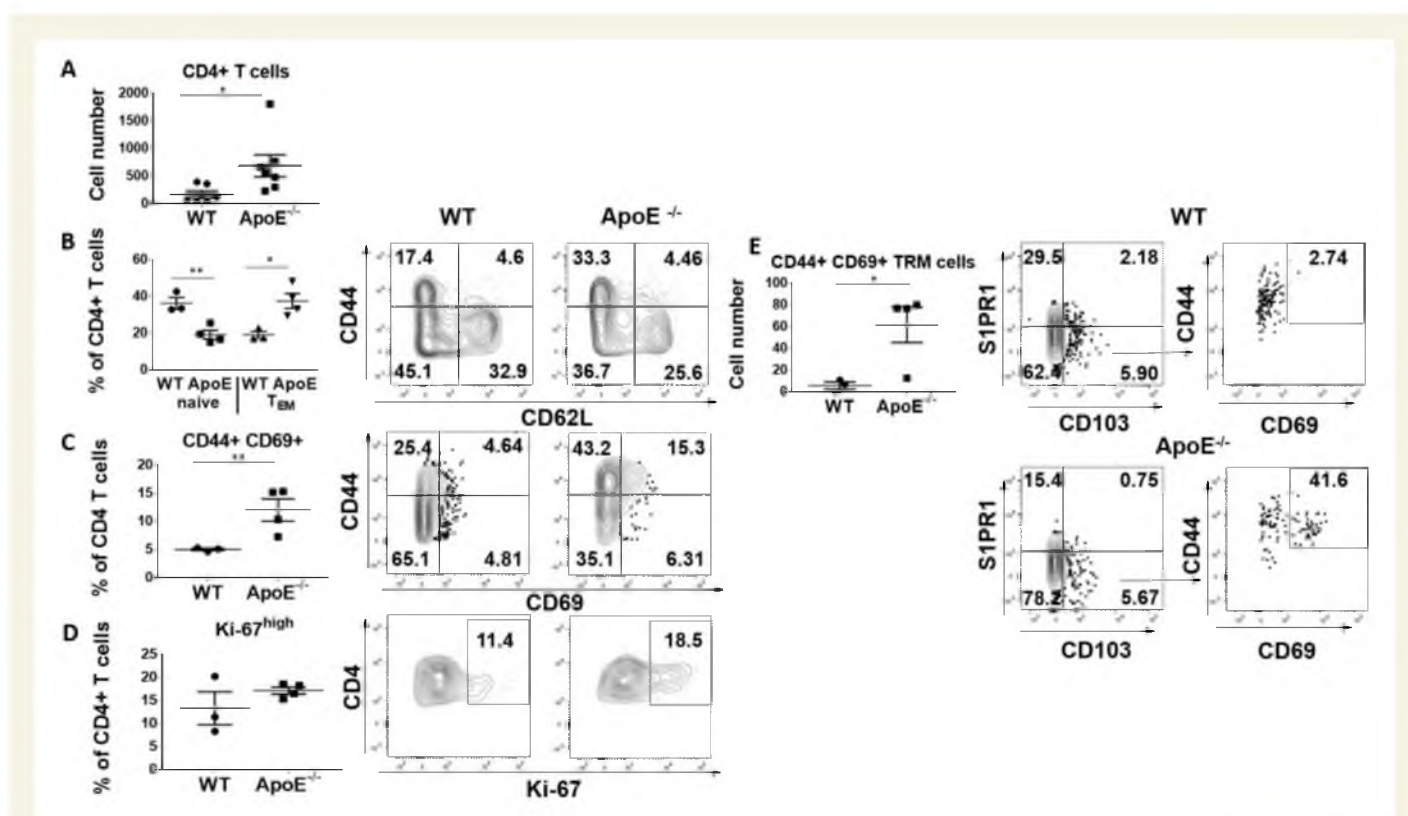
Having identified naïve T cells within the aorta, we investigated the mechanism of naïve T-cell homing from the circulation into aortic tissue. PSGL-1 is the major leucocyte ligand for P-selectin, while also showing

affinity for E- and L-selectin<sup>19</sup> but its role in vascular recruitment of naïve CD4<sup>+</sup> T cells remains unknown. To investigate this, we injected C57BL/6 WT mice with the anti-PSGL-1 antibody, 4RA10,<sup>20</sup> on two consecutive days and studied CD4<sup>+</sup> T-cell populations within the aorta 48 h following the first injection (Supplementary material online, Figure S8). Since PSGL-1 is common to all leucocytes, there was a decrease in the number of total leucocytes (>80%; 2.7-fold) in the vessel of anti-PSGL-1-treated animals (Supplementary material online, Figure S8A). A marked reduction was also identified for total CD4<sup>+</sup> T cells (88%; 4.8-fold; Supplementary material online, Figure S8B). Interestingly, both activated and naïve T cells were reduced by anti-PSGL-1 treatment with naïve CD4<sup>+</sup> T cells reduced by 96% which equated to a 5.7-fold reduction in this population (Supplementary material online, Figure S8C). Our data demonstrate that PSGL-1 has a key role in promoting naïve T-cell recruitment to the aorta.

### 3.4 Early stage atherosclerosis is associated with vascular T-cell expansion, activation and selective Th1 polarization

Finally, we sought to assess differences in the phenotype of CD4<sup>+</sup> T cells in the aorta, rLNs, and spleen of WT vs. apoE<sup>-/-</sup> mice. As already demonstrated by others,<sup>6</sup> apoE<sup>-/-</sup> aortas contained significantly more (~400%) CD4<sup>+</sup> T cells compared with WT (Figure 5A). In apoE<sup>-/-</sup> aortas, we observed a lower frequency of naïve CD4<sup>+</sup> T cells and a significant





**Figure 5** CD4<sup>+</sup> T cells show a more activated phenotype in atherosclerotic aortas. Wild-type and apoE<sup>-/-</sup> mice were maintained on a high-fat diet for 10–12 weeks. Mice were culled and aortic single cell suspensions assessed via flow cytometry for (A) CD4<sup>+</sup> T-cell number per vessel, (B) naïve (CD62L<sup>+</sup>CD44<sup>-</sup>) vs. effector memory (CD62L<sup>-</sup>CD44<sup>+</sup>) T-cell phenotype, (C) activation status, and (D) proliferation. (E) Additionally, aortas were stained for detection of T<sub>RM</sub> cells (S1PR1<sup>+</sup>CD103<sup>+</sup>CD44<sup>+</sup>CD69<sup>+</sup>) cells. (A) Data shown represent  $n = 6-7$  groups with each group comprising 3–8 pooled aortas from 6 to 7 independent experiments. (B–E) Data shown represent  $n = 3-4$  groups with each group comprising 6–8 pooled aortas from 3 to 4 independent experiments. Individual data points represent average value per group; horizontal bars denote mean. Results are presented as mean  $\pm$  SEM. The Student's unpaired t-test; \* $P < 0.05$ , \*\* $P < 0.01$ .

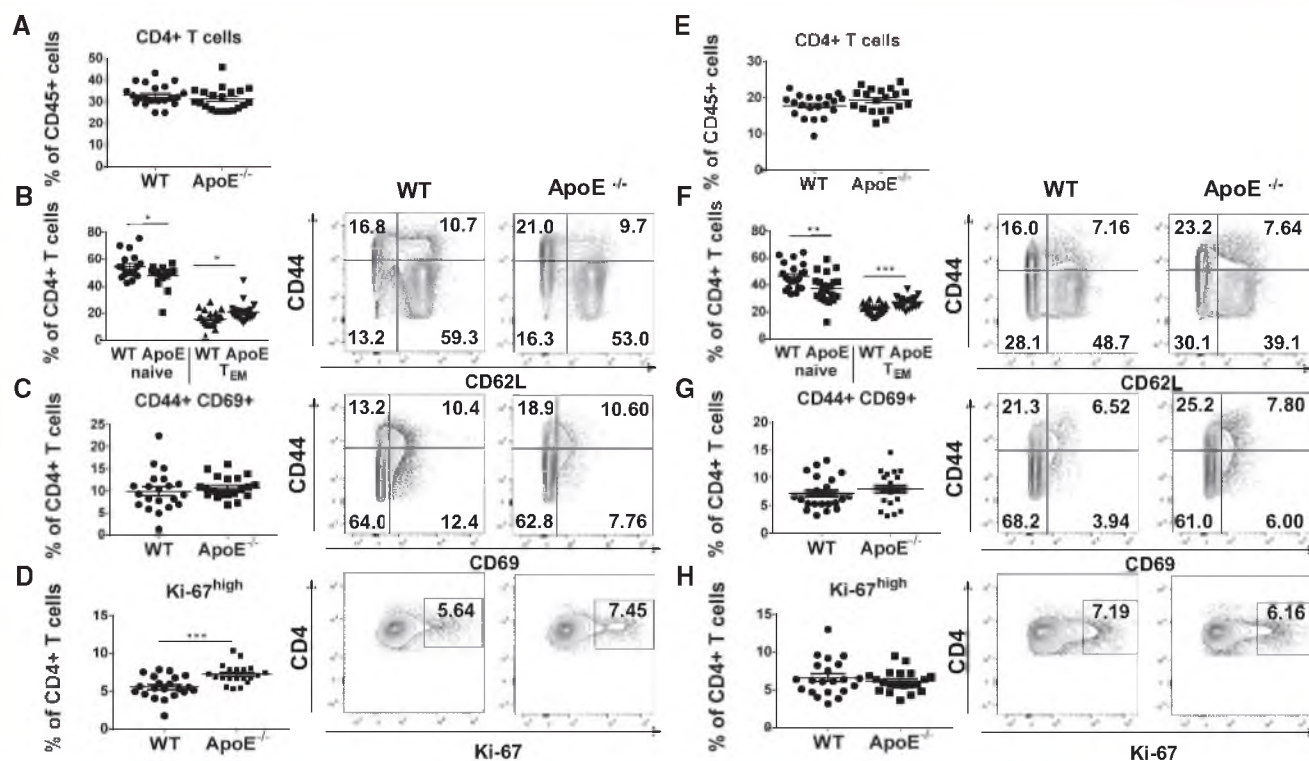
switch from naïve (CD62L<sup>+</sup>CD44<sup>-</sup>) to an effector memory (T<sub>EM</sub>) population (CD62L<sup>-</sup>CD44<sup>+</sup>) was noted (Figure 5B) concomitant with increased surface expression of the T-cell activation marker CD69 (Figure 5C). No significant difference was noted for T-cell proliferation (Figure 5D).

CD4<sup>+</sup> tissue-resident memory cells (T<sub>RM</sub>) have been identified in several tissues including lung<sup>21,22</sup> and skin<sup>23</sup> but have never been investigated in the vasculature. Using a well-defined marker profile for T<sub>RM</sub>, namely Sphingosine-1-phosphate receptor 1 (S1PR1)-CD103<sup>+</sup>CD44<sup>+</sup>CD69<sup>+</sup>, we identified a small population fitting the T<sub>RM</sub> profile in the aortas of apoE<sup>-/-</sup> mice and while the absolute number of cells were low, they were 10 times higher than that observed in WT aortas (Figure 5E).

On examination of rLNs and spleen (Figure 6), the CD4<sup>+</sup> populations were equivalent between WT and apoE<sup>-/-</sup> animals both for rLNs (Figure 6A) and spleen (Figure 6E). Both tissues showed a modest increase in T<sub>EM</sub> cells (~5%) in apoE<sup>-/-</sup> mice (Figure 6B and F, respectively) which is in contrast to the ~20% increase observed in apoE<sup>-/-</sup> aorta. Also, in contrast to the aorta, no increase in CD44<sup>+</sup>CD69<sup>+</sup> T cells was observed in the rLNs (Figure 6C) or spleen (Figure 6G). The cells of rLNs in apoE<sup>-/-</sup> mice did; however, show a higher rate of proliferation compared with WT, a phenotype not observed in the spleen (Figure 6D and H, respectively).

The major pro-atherosclerotic T-cell response in atherosclerosis is driven by Th1 cells.<sup>24</sup> We, therefore, employed direct *in vivo* intracellular cytokine staining to quantify T cells actively secreting Th1 cytokines without exogenous stimulation.<sup>25</sup> Following 12 week's high-fat diet (HFD), mice were injected with Brefeldin A 5 h before culling. CD4<sup>+</sup> T cells were assessed for the expression of the signature Th1 cytokine: IFN- $\gamma$  in addition to TNF- $\alpha$  (Figure 7A–C). There were ~10 times more IFN- $\gamma$ +CD4<sup>+</sup> T cells in apoE<sup>-/-</sup> aortas compared with WT. Aortic Th1 cells were not actively producing TNF- $\alpha$  (Figure 7A). No significant differences in the Th1 phenotype were observed in either rLNs or spleen (Figure 7B and C, respectively).

We considered that the increased frequency of CD44<sup>hi</sup> CD4<sup>+</sup> T cells in the apoE<sup>-/-</sup> spleen and rLNs may be associated with Treg polarization and expansion which is known to play a protective role in the pathology.<sup>24</sup> CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs were detected in both WT and apoE<sup>-/-</sup> aortas and although a trend towards less Tregs was observed in apoE<sup>-/-</sup> aortas, this did not reach significance (Figure 7D). This was in contrast to SLOs where Tregs formed a larger proportion of the total CD4<sup>+</sup> population with this value increasing in apoE<sup>-/-</sup> rLNs (Figure 7E) and spleen (Figure 7F). In summary, early stage atherosclerosis is associated with vascular T-cell expansion, activation and selective Th1 polarization, whereas SLOs are skewed towards enhanced Treg expansion as observed previously.<sup>26</sup>



**Figure 6** CD4<sup>+</sup> T-cell phenotype in renal lymph nodes and spleen of apoE<sup>-/-</sup> mice vs. wild-type. Wild-type and apoE<sup>-/-</sup> mice were maintained on a high fat diet for 10–12 weeks. Mice were culled and single cell suspensions from (A–D) rLNs to (E–H) spleen were assessed via flow cytometry for (A, E) CD4<sup>+</sup> T-cell frequency, (B, F) naïve vs. effector memory T-cell phenotype, (C, G) activation status, and (D, H) proliferation. Data shown represent  $n = 20$ –21 mice from 3 to 4 independent experiments. Individual data points represent average value per mouse; horizontal bars denote mean. Results are presented as mean  $\pm$  SEM. The Student's unpaired *t*-test; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

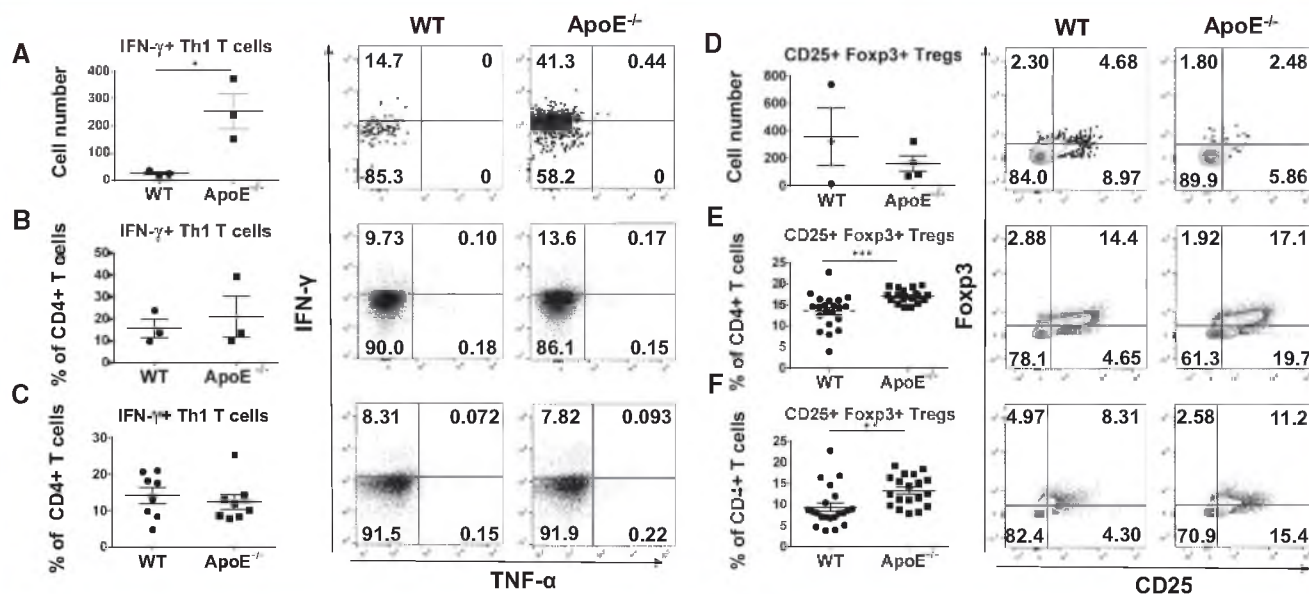
## 4. Discussion

We have conclusively identified naïve T cells within the aorta of several mouse strains. We also illustrated how the aorta supports local T-cell priming in a model system. Indeed, in OT-II mice, aortic CD4<sup>+</sup> T cells can be primed as efficiently as lymphoid T cells. Naïve T cells also depend, at least in part, on PSGL-1 for aortic homing. We have also performed a comparative assessment of the CD4<sup>+</sup> T-cell phenotype between the atherosclerotic aorta, an aortic draining lymph node and the spleen, highlighting the importance of the local aortic immune response. A significant Th1 response evolved in the aorta, whilst T cells in SLOs were biased more towards a regulatory phenotype. These data support the hypothesis that atherosclerosis induces local vascular Th1 cell responses and that this local response is quite distinct from the more tolerogenic responses observed in SLOs.

Naïve T cells have been identified in peripheral tissues including brain, pancreas, lung, skin, and testes.<sup>27</sup> Here, we demonstrate that naïve CD4<sup>+</sup> T cells constitutively reside in the aorta of WT mice. We found similar proportions of T cells amongst total leucocytes in both the adventitia and intima/media with naïve T cells being detected in both compartments. We confirmed by microscopy that T cells can be found bound to non-inflamed aortic endothelium and resided in close proximity to DCs indicating T cells may be able to enter the aortic wall from the lumen. Indeed, microvessels are generally absent in the healthy aortic wall,<sup>28</sup> which strongly suggests that the major point of entry in naïve

vessels is via the arterial lumen. The presence of naïve T cells within the intima/media is intriguing, given that the intima is the principal location of APCs in the naïve aorta<sup>7</sup> where they accumulate in the subendothelial space. Here, we have fully delineated the repertoire of myeloid cells within the naïve aorta by CyTOF analysis revealing 12 types of myeloid cells including multiple MHC-II<sup>hi</sup> APC populations. This offers the potential for naïve T cells to encounter APCs in the environment where lipid accumulation/oxidation and atherogenesis begins. Much of the evidence regarding local T-cell activation and clonal expansion directly in the vessel is circumstantial such as co-localization immunohistochemistry of atherosclerotic plaques<sup>3</sup> or the use of artificial systems *ex vivo*,<sup>8</sup> hence it is unknown if T-cell priming can occur in the aorta *in vivo*. We were able to demonstrate activation of aortic OT-II T cells following antigen with kinetics paralleling those observed for splenic OT-II cells with peak proliferation observed at 72 h. At 72 h, we also observed BrdU uptake, indicating cells were undergoing mitosis. This was following a 2-h pulse of BrdU, which would strongly indicate the BrdU<sup>+</sup> cells were of a local nature (i.e. in the target tissue of interest). To confirm this hypothesis, we pre-treated OT-II mice with FTY720 and blocking antibodies to P- and E-selectin prior to administration of antigen, thus ensuring any clonally expanded OT-II T cells within the aorta could only derive from endogenous naïve T cells encountering an aortic APC presenting ovalbumin peptide on MHC-II. The result from this experiment confirmed that priming of naïve CD4<sup>+</sup> cells occurred in the aortic wall and is direct evidence that APCs within the vessel can uptake and present antigen locally





**Figure 7** Atherosclerosis induces divergent effects on aortic vs. lymphoid tissue T-cell polarization. Wild-type and apoE<sup>-/-</sup> mice were maintained on a high-fat diet for 10–12 weeks. Mice were then separated into two experimental groups. Group 1 received 300 μg of Brefeldin A 5 h prior to culling and single cell suspensions of (A) aorta, (B) rLNs, and (C) spleen were utilized for intracellular staining of CD4<sup>+</sup> T cells for the Th1 cytokines: IFN-γ and TNF-α. Representative plots and graphs show IFN-γ<sup>+</sup> Th1 T cells. Plots are gated on CD4<sup>+</sup>TCR-β and results represent *n* = 3 per group with group containing three pooled aortas, three pooled rLNs, and *n* = 8 spleens from three independent experiments. In Group 2, mice were culled and single cell suspensions of (D) aorta, (E) rLNs, and (F) spleen were assessed via flow cytometry for the presence of CD4<sup>+</sup> Tregs (CD25<sup>+</sup>FoxP3<sup>+</sup>). Data shown for aorta represent *n* = 3–4 groups with each group containing 6–8 pooled aortas. Data shown for rLNs and spleen represent *n* = 20–21 individual organs. All data derived from 3 to 4 independent experiments. Individual data points represent average value per group/organ; horizontal bars denote mean. Results are presented as mean ± SEM. The Student's unpaired *t*-test; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

to CD4<sup>+</sup> T cells and induce clonal expansion. Using OT-II mice, we were able to visualize T cells and DCs forming cell contacts following antigen challenge. Such contacts were observable in the adventitia, media, and intima. Of interest, DCs and T cells were found to reside in areas of the media where muscle fibres were less dense. The observation that DCs and T cells can interact in all the aortic layers indicates that the architecture of the aortic wall can support local T-cell activation, even in the absence of a local inflammatory stimulus. Whilst priming of T cells in healthy WT aorta is very unlikely given the small polyclonal population that exists with respect of SLOs, the situation in atherosclerotic plaques may offer an environment more conducive to T-cell activation. In the context of atherosclerosis—in the presence of chronic pro-inflammatory stimuli—local antigen presentation could, in theory, take place within the developing lesion, where T cells and APCs are more highly concentrated with T cells co-localizing with activated DCs.<sup>2,3</sup> Evidence for aortic T-cell priming in atherosclerosis was previously suggested in apoE<sup>-/-</sup> mice by the fact that the T-cell repertoire within atherosclerotic aortas became more restricted over the course of pathology while no changes in T-cell clonality could be detected in SLOs.<sup>12</sup>

The mechanism by which naïve T cells enter the aorta is unknown. We identified PSGL-1, the major leucocyte ligand for endothelial selectins, as a receptor of interest in regulating homing of naïve T cells into the aorta; since we demonstrated that naïve T cells are depleted in the aorta following anti-PSGL-1 treatment. PSGL-1 blockade or deficiency

reduced atherosclerosis formation, adhesive interactions between endothelial cells and leucocytes, and neointima formation in apoE<sup>-/-</sup> mice.<sup>29–31</sup> Naïve T cells lack a fully glycosylated PSGL-1 so binding to selectins is lower than for activated T cells yet some binding affinity still remains.<sup>32</sup> Adoptively, transferred T cells have been previously shown to enter atherosclerotic aortas in a partially L-selectin dependent manner despite L-selectin receptors being absent from aortic tissue.<sup>6</sup> This apparent discrepancy can be reconciled by the fact that L-selectin expressed on an endothelial bound T-cell binds to PSGL-1 on an unbound T-cell (a process termed secondary capture).<sup>33</sup> The initial interaction (primary capture) involves a T-cell binding to P- or E-selectin on the vessel wall. In fact, it has been demonstrated that the absence of L-selectin has no effect on aortic leucocyte primary capture and rolling. Moreover, following 12 weeks HFD, neither T-cell number nor plaque area was altered between apoE<sup>-/-</sup> and apoE<sup>-/-</sup>/L-selectin<sup>-/-</sup> mice.<sup>34</sup>

We also noticed that T<sub>EM</sub> cells were less affected by PSGL-1 blockade (2.7-fold reduction) compared with naïve cells, making up a greater proportion of the CD4<sup>+</sup> cells in the treatment group compared with isotype. T<sub>EM</sub> cells may use additional receptors, to facilitate binding to endothelial selectins including: T-cell immunoglobulin and mucin domain 1 (TIM-1),<sup>35</sup> CD44,<sup>36</sup> and E-selectin ligand-1.<sup>37</sup> PSGL-1 on naïve T cells can also bind the chemokines, CCL19, and CCL21<sup>38</sup> but expression of these chemokines in vascular tissue is low or absent under non-inflammatory conditions.<sup>4,39,40</sup> Therefore, we consider these unlikely to contribute to the reduced trafficking observed in WT mice. There may

also be chemotactic factors in the vessel wall derived from myeloid cells that contribute to the reduced trafficking of naïve T cells observed after PSGL-1 blockade, as an indirect effect, due to concurrent reductions in other leucocyte subsets.

Finally, we performed a phenotypic analysis of CD4<sup>+</sup> T cells between WT and apoE<sup>-/-</sup> aortas, under similar HFD conditions. In line with previous results,<sup>6</sup> here, we show that aortic CD4<sup>+</sup> T cells display a near four-fold increase in numbers in apoE<sup>-/-</sup> mice compared with WT mice. We next investigated the relative proportions of naïve vs. activated CD4<sup>+</sup> T cells. Naïve CD4<sup>+</sup> T cells were significantly reduced in aortas of apoE<sup>-/-</sup> mice compared to WT, concomitant with an increase in T<sub>EM</sub> frequency. In contrast, when we examined rLNs and spleens, we discovered that the CD4<sup>+</sup> populations were equivalent in terms of magnitude with only a minor switch towards an activated phenotype, considerably less than what was observed in the aorta. This is consistent with a lack of increased proliferation of splenic CD4<sup>+</sup> T cells previously observed at 8 weeks HFD in apoE<sup>-/-</sup> mice.<sup>41</sup>

Th1 cells are pro-atherosclerotic in both humans<sup>3,42,43</sup> and animal models.<sup>24</sup> By employing intracellular cytokine staining to reveal *in vivo* cytokine prolif, we showed that IFN- $\gamma$ <sup>+</sup> (Th1) T cells were 10 times more abundant in apoE<sup>-/-</sup> aortas. In contrast, we did not detect significant differences in the Th1 population in either the rLNs or spleen. We also quantified CD4<sup>+</sup> Tregs, which are known to be atheroprotective in animal models.<sup>24</sup> Tregs were a small proportion of total CD4<sup>+</sup> T cells within the aorta with no significant differences between WT and apoE<sup>-/-</sup> mice. In contrast, Tregs grew as a proportion of CD4<sup>+</sup> T cells both in rLNs and spleen. In support of this data, a study conducted on Foxp3-eGFP/LDLr<sup>-/-</sup> mice showed a progressive increase in the frequency of splenic CD4<sup>+</sup> Tregs at 4, 8, and 20 weeks HFD, while the total CD4<sup>+</sup> T-cell population remained unchanged.<sup>26</sup>

The immune-mechanisms that drive aortic T-cell activation and Th1 response in apoE<sup>-/-</sup> mice are likely to be multi-factorial with both antigenic and non-antigenic stimuli contributing to the aortic resident T-cell phenotype. One additional factor worth considering is the potential presence of B cell follicles, resembling early tertiary lymphoid organs, which can be found in even young apoE<sup>-/-</sup> mice<sup>44</sup> and these structures, if present, may exert immunomodulation on the underlying vascular T-cell response.

We have used a model *in vivo* system to illustrate the capacity that the naïve aorta has to promote local T-cell priming. Other approaches that could have further validated these findings such as orthotopic aortic transposition utilizing an aortic graft from a donor with trackable (i.e. fluorescent) cells could also have aided in discriminating local from systemic immune effects. However, not only are such approaches technically challenging, the grafts would yield a very low number of T cells with respect to an entire intact aorta thus necessitating a large number of surgeries to produce sufficient tissue for quantifiable data. Future studies, however, utilizing such approaches coupled with detailed temporal phenotyping of TCR usage in experimental atherosclerosis would further enhance our knowledge of when and where antigenic stimulation of T cells occurs.

In conclusion, the aorta can support T-cell priming and local activation of CD4<sup>+</sup> T cells is associated with the vascular specific Th1 response we observed in early stage atherosclerosis in the apoE<sup>-/-</sup> aorta.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

**Conflict of interest:** none declared.

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